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The RNA Component of Human Telomerase

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Eukaryotic chromosomes are capped with repetitive telomere sequences that protect the ends from damage and rearrangements. Telomere repeats are synthesized by telomerase, a ribonucleic acid (RNA)-protein complex. Here, the cloning of the RNA component of human telomerase, termed hTR, is described. The template region of hTR encompasses 11 nucleotides (5'-CUAACCCUAAAC) complementary to the human telomere sequence (TTAGGG)_n. Germline tissues and tumor cell lines expressed more hTR than normal somatic cells and tissues, which have no detectable telomerase activity. Human cell lines that expressed hTR mutated in the template region generated the predicted mutant telomerase activity. HeLa cells transfected with an antisense hTR lost telomeric DNA and began to die after 23 to 26 doublings. Thus, human telomerase is a critical enzyme for the long-term proliferation of immortal tumor cells.

Human chromosomes terminate with several kilobases of the simple telomere repeat (TTAGGG)_n. Telomeres protect the chromosomes from DNA degradation, end-to-end fusions, rearrangements, and chromosome loss (1, 2). Because DNA polymerases synthesize DNA in the 5' to 3' direction and require an RNA primer for initiation, telomeric DNA may be lost at chromosome ends unless the termini are specifically extended by telomerase (3, 4). Telomerase is a specialized ribonucleoprotein polymerase that contains an integral RNA with a short template element that directs the synthesis of telomeric repeats at chromosome ends (5, 6).

The deregulation of telomerase may participate in cellular immortality and oncogenesis. Normal human somatic cells express low or undetectable telomerase activity and progressively lose their telomeric sequences with replicative senescence in vitro or with normal, in vivo aging (7-14). In contrast, germline cells and almost all tumor cell lines and tissues express telomerase and maintain telomere length through an indefinite number of cell divisions (12, 15-17). Transfection of normal somatic human cells with viral oncogenes leads to cell division beyond the normal senescence checkpoint and the continued

loss of telomere sequences, defined as "crisis." At crisis, telomeres are critically short and genomic instability is marked (12, 15, 18). Although most cells die in crisis, the rare cells that escape this fate are typically aneuploid, express telomerase activity, and have a stable telomere length. Therefore, telomerase activation can be a critical step in cell immortalization.

Short telomeres have been detected in a range of tumor cell lines and tissues, including breast cancer, Wilms tumor, colorectal carcinoma, and leukemia (8, 19-26), which suggests that telomere shortening may be involved in oncogenesis. Perhaps because of this telomere shortening, over 90 percent of primary human tumors express telomerase activity (17, 27). Thus, telomerase may be activated in the premalignant cell as telomeres get critically short, and continued telomerase activity may be required for the long-term growth of the fully malignant cancer cell (7-9, 12, 17). We have cloned the RNA component of human telomerase and show here that expression of an antisense telomerase RNA leads to telomere shortening and cell death.

Cyclic selection cloning of telomerase RNA candidates. The RNA component of telomerase has been cloned from many ciliates (28-30) and yeast (31). No similarity in RNA size or sequence was found between these species, which indicates that the primary sequence of telomerase RNAs is not conserved. Conventional low-stringency hybridization techniques, therefore, were unlikely to yield the human telomerase RNA. In *Tetrahymena*, which contains >20,000 telomeres, telomerase RNA is

present at ≈20,000 copies per cell (32). Because human cells have only 92 telomeres and telomerase activity in humans is weaker than in *Tetrahymena* (33), we anticipated that the human telomerase RNA might be a rare transcript requiring special protocols to enrich for this RNA.

Enrichment for telomerase genes was initially carried out by subtractive hybridization or by partial telomerase purification (34). RNA was prepared from telomerase-positive cells (293), telomerase-negative cells (IMR90), or partially purified telomerase fractions from 293 cells. Complementary DNA (cDNA) libraries were generated from these RNAs by random-primed reverse transcription of the RNAs and the addition of synthetic linkers to allow for polymerase chain reaction (PCR) amplification. Subtraction of the telomerase-negative library from the telomerase-positive library generated the telomerase-enriched subtracted PCR-cDNA library. In addition, a purified PCR-cDNA library was prepared from partially purified telomerase enzyme fractions, which represented a second independent library enriched for potential telomerase RNAs.

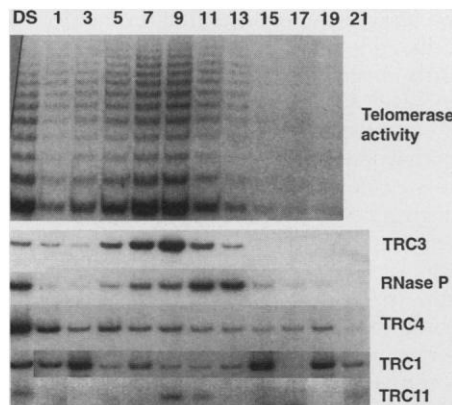
Telomerase RNAs typically contain 1.5 repeats of the telomere complementary sequence (29, 30, 35). We designed a PCR cyclic selection method to enrich for candidate telomerase RNAs containing the predicted human telomeric template sequence CUAACCCUA. Both the subtracted and purified PCR-cDNA libraries were enriched for telomerase template-containing sequences by hybridization to a biotinylated 12-nucleotide complement to the predicted template (36). After hybridization, cDNAs bound to the biotinylated oligonucleotide were separated from nontemplate cDNAs with the use of streptavidin-agarose magnetic beads. To maximize the concentration of cDNAs containing CUAACCCUA in the libraries, we used three or four repetitive cycles of positive selection with the biotinylated, template-specific oligonucleotide. After the third cycle of selection, individual PCR bands could be resolved on native polyacrylamide gels, which indicated that some sequences had been highly selected. Individual bands were excised, subcloned into pBluescript, and sequenced. Twelve cDNAs containing the predicted telomere template (CUAACCCUA) were identified as telomerase RNA candidates (TRCs).

Preliminary identification of TRC3 as the RNA component of human telomerase. To determine which of the 12 candidates might encode the human telomerase RNA, we initially assayed for the inhibition of telomerase activity using antisense oligonucleotides. *Tetrahymena*, *Euplotes*, and *Oxytricha* telomerases are specifically inhibited by oli-

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Fig. 1. TRC3 RNA correlates with biochemical purification of telomerase activity. S100 extracts from 293 cells were fractionated over DEAE-Sepharose and then Sephacryl S300HR resins. Fractions containing active telomerase were pooled (DS input) and then loaded onto a preformed glycerol gradient (15 to 42 percent). After centrifugation, gradient fractions were collected (fraction 1 = bottom) and assayed for telomerase activity by the PCR method (17). RNA was extracted from each fraction, and cDNA was made with random hexamers as primers. The resulting cDNA was included in PCR reactions with the use of sequence-specific oligonucleotides derived from the TRC candidates or from RNase P (55) as indicated. The reaction products were resolved by polyacrylamide gel electrophoresis.



gonucleotides that are complementary to the telomerase template and adjacent sequences (28–30). Therefore, we designed oligonucleotides that would hybridize to the template and adjacent regions for each of the candidate clones. Extracts with telomerase activity were then tested for their ability to elongate each of the antisense oligonucleotides and the nontelomeric telomerase primer TS [oligonucleotide AATCCGTCGAGCAGAGTT, which is known to serve as an effective telomerase primer (17)]. The oligonucleotides complementary to one of the candidates, TRC3, inhibited telomerase activity, whereas oligonucleotides designed for the other 11 candidates did not.

In ciliates, the pattern of inhibition by antisense oligonucleotides that were based on the telomerase RNA component is dependent on their targeting to specific sequences around the telomeric template element (28–30). Therefore, we designed a series of oligonucleotides complementary to various sequences near or distal to the putative template of TRC3. These oligonucleotides were again tested for their ability to inhibit telomerase activity (37). The data indicated that antisense inhibition of human telomerase requires oligonucleotide complementarity to sequences in and around the TRC3 template and that antisense oligonucleotides distal from the tem-

plate were not inhibitory, which is similar to the pattern observed in the ciliates.

An additional test of the TRC clones was copurification with telomerase activity. Telomerase activity was partially purified, and fractions were tested for the TRC RNAs by reverse transcriptase (RT)-PCR (Fig. 1). Telomerase activity peaked in fractions 7 to 9 of the glycerol gradient. Of the candidates tested, only TRC3 had a signal that correlated with telomerase activity; ribonuclease (RNase) P and three other TRC candidates did not copurify with telomerase. Similar copurification of TRC3 with telomerase activity was observed in a different purification scheme (38). Thus, both copurification and the antisense inhibition experiments suggested that TRC3 encodes the human telomerase RNA component.

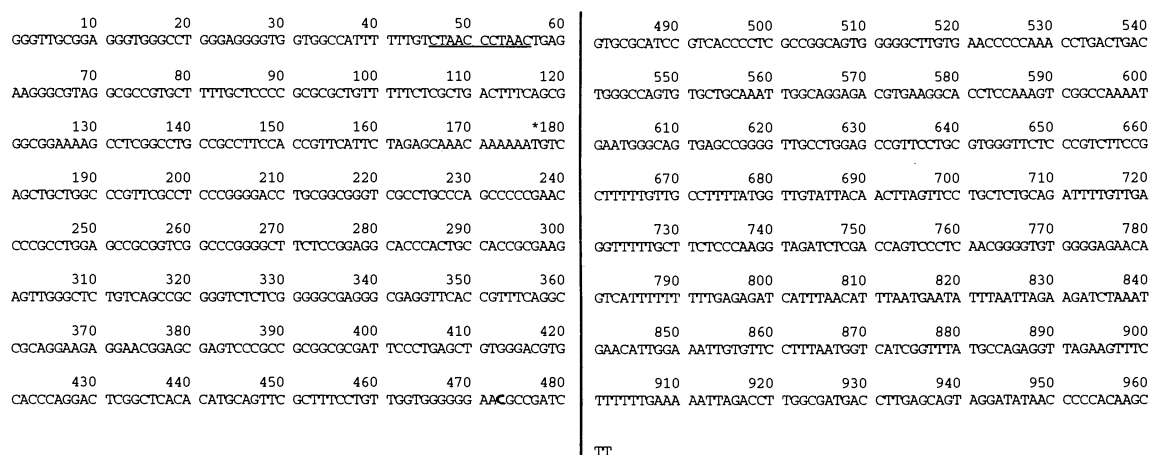
Southern (DNA) blot analysis and in situ hybridization demonstrated that TRC3 is a single-copy gene localized to the distal quarter of the long arm of chromosome 3 (39). TRC3 cDNA was used to probe a human genomic DNA library (lambda ZAP II; Stratagene). A single positive clone with a 15-kb insert was isolated and found to contain an exact copy of the TRC3 cDNA fragment used to probe the library. The restriction pattern deduced from the TRC3 Southern blot was consistent with the restriction pattern observed in the TRC3-

selected lambda clone, which confirmed that this lambda clone contained TRC3 genomic DNA (39).

Mutation of the TRC3 template. Alteration of the template region of *Tetrahymena* and yeast telomerase RNA genes leads to corresponding changes in the telomere repeat synthesized (6, 31, 35). To determine if alteration of the TRC3 template region would produce a similar result, a genomic DNA fragment that expresses the entire TRC3 gene sequence was subcloned from lambda and mutagenized. The 2.6-kb Hind III-Sac I fragment containing TRC3 was first inserted into a modified version of pBluescript, generating the genomic TRC3 plasmid pGRN33. The full TRC3 sequence was then obtained from this genomic subclone (Fig. 2). The 5' and 3' ends of the RNA transcript were mapped with 5' primer extension PCR and RT-PCR (40). The size of the RNA transcript was ≈ 450 bases, which is consistent with its migration on Northern (RNA) blots. The transcribed region for TRC3 was central to the hTR gene fragment in pGRN33 and contains 1.4 kb of upstream sequence.

The predicted template sequence of TRC3 was altered from CUAACCCUA to CCAACCCCA (MuC) or CAAACCCAA (MuA) by in vitro mutagenesis (41) of the genomic plasmid pGRN33. If incorporated into functional telomerase, these mutant RNAs should direct the synthesis of TTGGGG (MuC) or TTTGGG (MuA) rather than that of the wild-type repeat TTA-GGG. These mutant telomerase activities could be distinguished from that of the wild-type because they no longer require deoxyadenosine triphosphate (dATP) for activity and only the wild-type activity is sensitive to termination by dideoxyATP (ddATP). A double mutant (MuC*) was also prepared in which a 17-bp insertion was present at +176 bp (asterisks in Figs. 2 and 3A) in addition to the MuC template. This mutant allowed specific detection of the altered RNA with a probe to the 17-bp insertion.

Fig. 2. Sequence of hTR and the downstream region. Both strands were sequenced from the genomic clone by the Sanger dideoxy method (56). The telomerase template is underlined, and the position of the 17-base insert (ATCG-GATCCAGACGAGC) in MuC* is shown at nucleotide 176 by an asterisk.



In preliminary experiments, we determined whether the 2.6 kb of genomic sequence was sufficient for expression of TRC3 *in vivo*. Cells were transiently transfected with the MuC* plasmid, and RNA expressed from the DNA was detected by RT-PCR with the 17-bp insert sequence as the primer in the reverse transcription step. The RNA was detected in MuC*-transfected cells but not in mock-transfected cells (42), which indicates that the 2.6-kb genomic clone was sufficient for TRC3 expression. Stable transformants were then derived by electroporation of MuC*, MuC, or MuA plasmids into HT1080 cells along with pCMVneo as a selection marker. Resistant clones were selected in G418, and expression of the mutant RNAs was verified by RT-PCR (43).

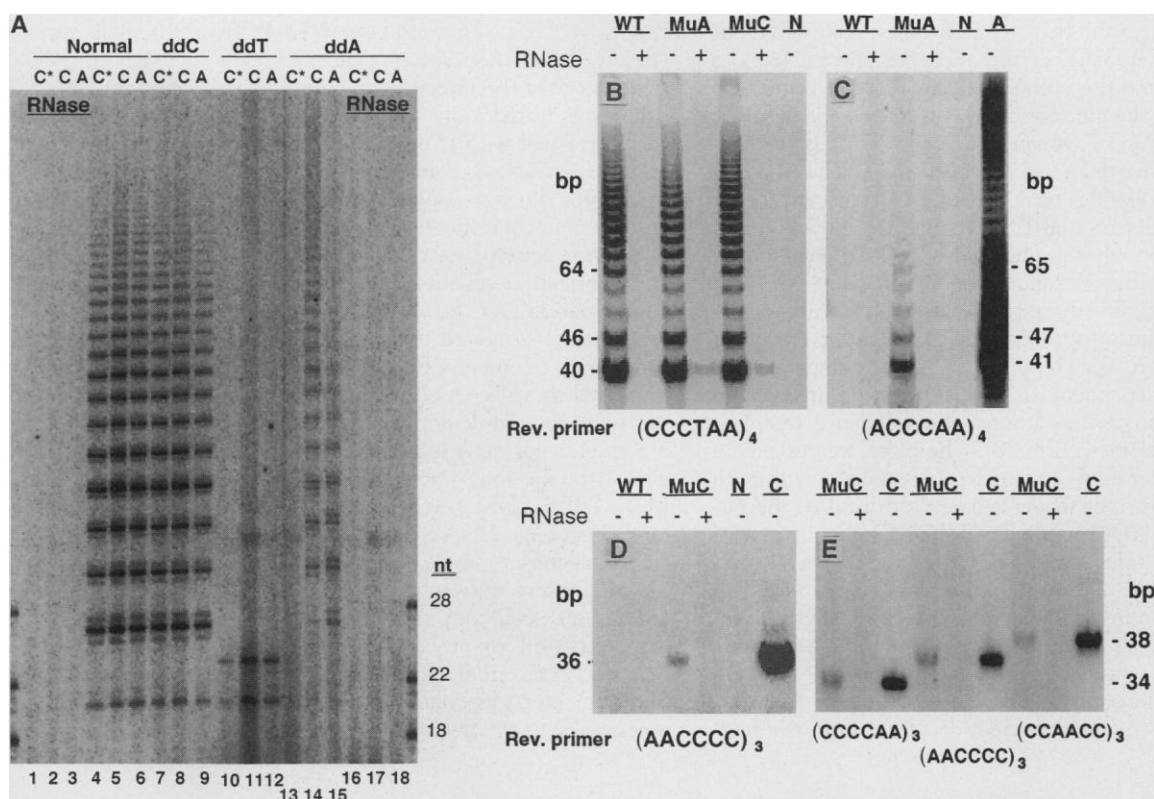
To test for mutant telomerase activity,

we assayed the extracts from untransfected cells and from three stable transformants with integrated MuC*, MuC, or MuA vectors (C*, C, or A in Fig. 3A). Because the mutant extracts were expected to contain both wild-type and mutant telomerase activities, various assay conditions were used to distinguish between them. Under normal reaction conditions [with deoxythymidine triphosphate (dTTP), [α -³²P]deoxyguanosine triphosphate (dGTP), and dATP], all three extracts from the mutant construct series showed telomerase activity that was sensitive to RNase. As expected, this activity was unaffected when dideoxycytosine triphosphate (ddCTP) was included in the reactions and was abolished by dideoxyTTP (ddTTP). In contrast, when ddATP was substituted for dATP, the C and A extracts still displayed RNase-sensitive telomerase

activity, whereas the C* extract did not. Assays of extracts from untransfected cells gave the same result as C*. The simplest interpretation of these results is that the ddATP-resistant activities represent telomerase reprogrammed with MuC or MuA TRC3 RNA. In contrast, the 17-bp insertion in C* inhibits reconstitution, which indicates that telomerase reconstitution is sensitive to certain alterations in the TRC3 sequence.

To confirm that the sequence synthesized by the mutant MuA was (GGGTTT)_n, we modified the existing PCR methodology for amplifying telomerase repeats (Fig. 3, B to E). Using synthetic telomerase products, we identified reaction conditions where a 3' return primer with the sequence d(C-CCAAACCCAAACCCAA) would ampli-

Fig. 3. Telomerase activity from cells expressing template-mutated TRC3. **(A)** Extracts from mutant TRC3-expressing stable transformants were fractionated by DEAE-Sepharose and were assayed for telomerase activity by conventional assays under various reaction conditions. Extracts from cells expressing MuC* TRC3 (lanes 1, 4, 7, 10, 13, and 16, labeled C*), MuC TRC3 (lanes 2, 5, 8, 11, 14, and 17, labeled C), or MuA TRC3 (lanes 3, 6, 9, 12, 15, and 18, labeled A) were assayed under normal reaction conditions (lanes 1 to 6), normal plus 0.5 mM ddCTP (lanes 7 to 9), normal minus dTTP plus 0.5 mM ddTTP (lanes 10 to 12), or normal minus dATP plus 0.5 mM ddATP (lanes 13 to 18). Assay reactions in lanes 1 to 9 contained 8 μ M total dGTP, of which a portion was 1 μ M [α -³²P]dGTP (800 Ci/mmol). To facilitate mutant telomerase detection, assay reactions in lanes 10 to 18 contained 8 μ M total dGTP, of which a portion was 2 μ M [α -³²P]dGTP (800 Ci/mmol). Extracts were treated with DNase-free RNase (25 μ g/ml for 10 min at 30°C) before telomerase assays (lanes 1 to 3 and 16 to 18). Flanking lanes contain DNA markers with sizes in nucleotides (nt) as indicated. **(B to D)** The second strategy for analyzing reprogramming of telomerase with template-mutated TRC3 was to use PCR primers specific for the repeat sequence (TTTGGG)_n, (TTGGGG)_n, or (TTAGGG)_n found in MuA, MuC, or wild-type (WT) TRC3, respectively. In (B), the presence of wild-type telomerase activity was detected in cell extracts from wild-type, MuA, and MuC cells in the presence of all four deoxynucleoside triphosphates (dNTPs). In (C), cell extracts were first incubated with the TS substrate in the presence of only dTTP and dGTP for 10 min at room temperature to allow for the addition of telomeric repeats. We then destroyed residual telomerase activity by boiling the extracts for 5 min. Telomerase products with specific DNA sequence were then detected by PCR amplification with the appropriate reverse (Rev.) primers and with all four dNTPs



and trace amounts of [α -³²P]dCTP. In lane N, no telomerase extract was added. As a control for MuA or MuC specificity, the synthetic telomerase products A [AATCCGTCGAGCAGAGTT-TGG (TTTGGG)₄TTTG] or C [ATCCGTCGAGCAGAGTT-GGGG(TTGGGG)₄TTGG] were prepared. To detect the MuA or MuC products, we used the reverse primers (ACCCAA)₄ or (AACCCC)₃ and found them to be specific for the MuA or MuC product. In further tests of the specificity of our PCR amplification conditions, synthetic A or C telomerase oligonucleotides generated the appropriate 6-nt ladder PCR products with (ACCCAA)₄ or a single band with the (CCCCAA)₃ reverse primer, respectively, whereas (TTAGGG)_n oligonucleotides did not produce any PCR products with the (ACCCAA)₄ or (CCCCAA)₃ reverse primers. **(E)** To confirm the sequence of the MuC products, we used three reverse primers: (CCCCAA)₃, (AACCCC)₃, and (CCAACC)₃, respectively, which gave PCR products with the corresponding mobility shifts. This result is representative of the predicted sequence of the MuC telomerase products and is further supported by the comigration of MuC products with the synthetic C products with each permutation of the reverse primer.

fy only (TTTGGG) $_n$ repeats and would not amplify repeats containing either wild-type (TTAGGG) $_n$ or MuC (TTGGGG) $_n$ (44). Under these conditions, extracts from MuA but not from wild-type cells generated products in the modified telomerase assay (Fig. 3C), which indicates that telomerase from MuA-containing cells generated (TTTGGG) $_n$ repeats that were similar to synthetically made (TTTGGG) $_n$ repeats ("A" in Fig. 3C).

These methods were also used to analyze the MuC mutant (Fig. 3D), which synthesized (TTGGGG) $_n$ repeats but typically generates a single band during the PCR amplification, as does synthetic (TTGGGG) $_n$ ("C" in Fig. 3D). The sequence of the MuC product was further confirmed by permutation of the MuC return primer (CCCCAA) $_3$ in the PCR reaction by two or four nucleotides (Fig. 3E). The MuC product was shifted in register by two or four nucleotides, respectively, as was the case with synthetically made (TTGGGG) $_n$ oligomer. This confirms the sequence of the MuC products. Taken together, these results verify that TRC3 encodes the RNA component of telomerase. Thus, TRC3 was renamed hTR for human telomerase RNA.

Expression of hTR. The *Tetrahymena*, *Euplotes*, and *Oxytricha* telomerase RNAs are apparently transcribed by RNA polymerase III (6, 28–30). However, the pres-

ence of runs of T residues within the transcribed region of hTR (Fig. 3) and preliminary results indicating sensitivity to α -amanitin (38) suggest that hTR might be an RNA polymerase II transcript. To determine if hTR is polyadenylated [poly(A) $^+$], poly(A) $^+$ transcripts were selected on oligo(dT) beads (38). The hTR remained in the poly(A) $^-$ fraction, whereas the message for glyceraldehyde phosphate dehydrogenase (GAPDH) was enriched in the poly(A) $^+$ fraction, which indicates that hTR is not polyadenylated. The lack of polyadenylation and apparent transcription by RNA polymerase II suggests that hTR may be regulated by the specialized transcription complex characteristic of small nuclear RNA promoters (45). Of the small RNAs characterized to date, only U3 has been reported to be transcribed by different RNA polymerases in different species (46, 47). This putative difference in transcription may reflect the complexity of regulation of telomerase in mammals compared to that in the ciliates.

Telomerase RNA is divergent between species. The length of the mature hTR transcript is approximately 450 nucleotides, which differs from that in both the ciliates [153 to 192 nucleotides (nt)] and *Saccharomyces cerevisiae* (1.3 kb). Little or no identity was found between the primary RNA sequence of hTR and that of the ciliates or

S. cerevisiae. Finally, comparison of hTR with the mouse homolog (48) reveals blocks of conserved regions with little similarity around the template. In contrast, the ciliate telomerase RNAs are conserved in the template region (30).

Testes and most cancer cells have telomerase activity, whereas in most normal somatic human cells in adults telomerase is not detected (17). To determine if hTR RNA is elevated in testis tissue and immortal cancer cell lines, we analyzed hTR and GAPDH transcripts from five mortal primary cell strains that lack detectable telomerase activity and from five immortal cancer cell lines and testis tissue that have high telomerase activity. The steady-state concentrations of hTR transcripts were two- to sevenfold greater in the tumor cell lines than in primary cells when normalized to GAPDH messenger RNA (Fig. 4A). Although higher concentrations of hTR were

Fig. 4. Expression of hTR in normal and immortalized cells and normal tissues. **(A)** The steady-state level of hTR and GAPDH RNA was determined with quantitative RT-PCR (43). Controls show that all PCR quantitations were in the linear range up to 23 cycles and that the signal was abolished if reverse transcriptase was left out (right two lanes). RT-PCR was analyzed for five normal telomerase-negative cell lines (lanes 1 to 5) and five tumor telomerase-positive cell lines (lanes 6 to 10): lane 1, primary fetal lung; lane 2, primary fetal hand skin; lane 3, adult primary prostate; lane 4, primary sinovial fibroblasts; lane 5, foreskin fibroblasts; lane 6, melanoma LOX; lane 7, leukemia U25x; lane 8, NCIH23 lung carcinoma; lane 9, colon tumor SW620; lane 10, breast tumor MCF7; lane 11, testes; lane 12, breast tumor MCF7 without RT; and lane 13, colon tumor SW620 without RT. PCR products were labeled with 32 P, resolved by 6% polyacrylamide gel electrophoresis, and quantified with a PhosphorImager (Molecular Dynamics). Relative steady-state transcription was normalized to GAPDH as a control for loading and is shown below each lane. Testis tissue was arbitrarily given the value of 100 to compare these data with those from the Northern blot in (B). Note that testis tissue typically expresses one-third of the GAPDH with equal RNA loading, so that this threefold difference was factored into our normalization value for testis tissue. **(B)** Northern RNA blot of hTR RNA prepared from human tissues (56). Telomerase RNA was detected in all tissues, with the highest expression seen in testis and ovary tissue. As a control for loading, the blot was washed and reprobed for 18S ribosomal RNA. The Northern hybridization signals were quantified on a PhosphorImager, and the hTR was normalized to the 18S loading control. The relative signal is indicated below the bands with testis tissue again given the value of 100.

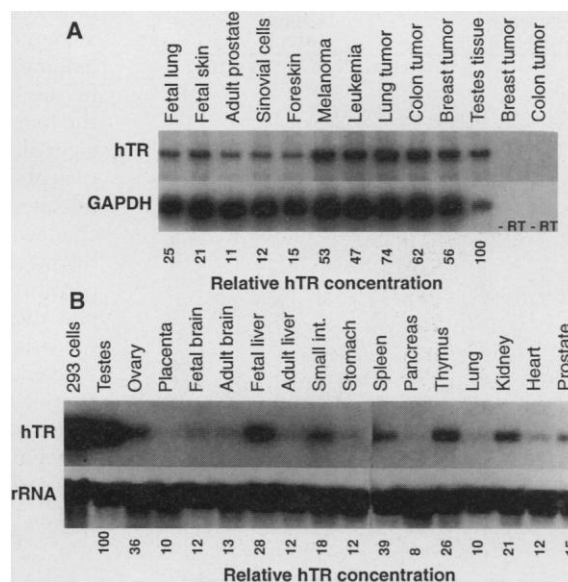


Table 1. Eleven to 18 stable clones were isolated from each transfection series. Clones from all isolates displayed identical morphology and growth profiles until 20 PDs, at which point the growth of most of the antisense-expressing clones (p10-3-hTR and pBBS-hTR) slowed. By PDs 23 to 26, these cells underwent crisis, characterized by the appearance of enlarged and rounded cells. However, not all of the clones generated from the pBBS-hTR-transfected HeTe7 cells underwent crisis; eight clones expressing antisense hTR continued growing in a manner similar to that of the control cultures. Cells from the clones were harvested at PD 23, and the average mean TRF lengths were determined. The *P* values were calculated by unpaired *t* test. As further controls for these experiments, antisense hTR or pBBS vector only were transfected into normal human foreskin BJ fibroblast cells at PD 22. None of the foreskin clones underwent crisis even after 38 doublings (PD 22 to PD 60). Replicative senescence for the BJ line typically occurs at >PD 90. The TRF analysis on the foreskin clones was done at PD 60. For each series, the ratio of clones that underwent crisis is indicated in relation to the total. C, control.

Plasmid	Crisis	TRF size		Ratio
		Mean	Short	
Experiment 1				
p10-3	No	—	—	0/7
p10-3-hTR	Yes	—	—	18/18
Experiment 2				
p10-3	No	3.30 ± 0.14	2.96	0/11
p10-3-hTR	Yes	2.40 ± 0.13	1.59	11/11
Experiment 3				
pBBS	No	3.22 ± 0.11	2.61	0/13
pBBS-hTRa	Yes	2.23 ± 0.19	1.71	4/4
pBBS-hTRb	No	3.03 ± 0.20	2.46	0/8
Control				
None	No	3.15 ± 0.09	2.96	C
Foreskin control				
pBBS	No	7.5		0/48
pBBS-hTR	No	7.5		0/36
None	No	7.5		C

present in the immortal cancer cells that expressed high telomerase activity, small but readily detectable amounts of hTR RNA were also present in mortal primary cells that lacked detectable telomerase activity. Testis tissue, which has high telomerase activity, had large amounts of hTR RNA, as expected.

Expression of hTR was also examined in a variety of normal human tissues by Northern blot analysis. Ovary tissue had large amounts of hTR, though not as large as that found in testis tissue. However, a number of other tissues also expressed hTR (Fig. 4B). These include normal kidney, prostate, and adult liver tissue, all of which lack detectable amounts of telomerase activity (17). These results confirm the data from cell lines (Fig. 4A) and indicate that telomerase RNA is present in a number of human tissues. Because telomerase activity is not detected in most somatic tissues, the presence of hTR suggests (barring assay failure) that hTR is inactive in these tissues. Similar tissue-specific differences in telomerase RNA expression can be seen in mouse tissues (48). However, many normal mouse tissues have telomerase activity (49, 50). Thus, telomerase activity parallels telomerase RNA concentrations in mice, but not in humans. Perhaps the protein components of telomerase are stringently regulated in human but not mouse cells, which

could explain why mouse cells spontaneously immortalize in culture, whereas human cells do not (51).

Expression of antisense hTR transcripts in HeLa cells. To examine the function of telomerase in an immortalized cell, we introduced antisense hTR expression constructs into HeLa cells (52). In parallel, control expression vectors lacking the antisense hTR coding sequence were also electroporated into HeLa cells. Clones containing antisense or control plasmids were selected in three separate experiments (Table 1). Initially, the 41 cultures expressing antisense hTR grew the same as those of cells with the control vector. However, at 23 to 26 population doublings (PDs) after transfection, 33 out of 41 antisense-expressing cultures underwent crisis (Fig. 5A). In contrast, none of the control clones (HeTe7 with vector alone or the telomerase-negative foreskin cells with hTR antisense expression) went into crisis.

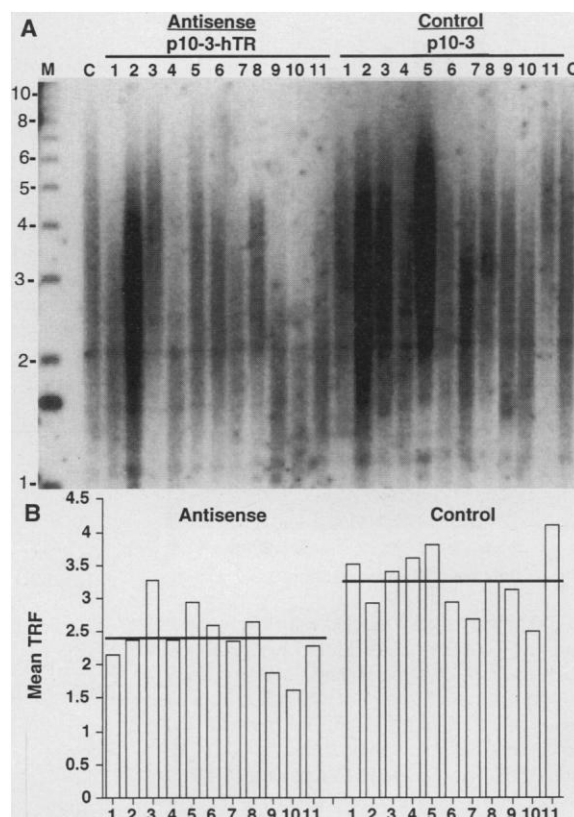
Cell crisis in the hTR antisense HeTe7 cultures was characterized by a marked inhibition in cell growth from 20 to 26 PDs and then the rounding up and detachment of cells from the plate over a period of 1 week. In 28 out of 33 cases in which the cells underwent crisis, rare (<1 percent) revertant colonies were observed when cultures were maintained for 3 weeks after the crisis event. The revertants may represent

variants that escape the inhibiting effect of the antisense hTR construct. In contrast to the antisense clones, none of the vector control cell lines had any change in growth or mortality over 50 doublings. In addition, normal young foreskin fibroblasts with long telomeres and no detectable telomerase showed no effect of the antisense constructs for at least 38 doublings after transfection.

To determine if telomere length and telomerase inhibition correlated with cell crisis in the antisense-expressing clones, we assayed telomere length (11) and telomerase activity (17) in several precrisis control and experimental colonies at 23 PDs after transfection. All colonies containing the control vector had mean TRF (terminal restriction fragment) lengths (3.30 and 3.22 kb for experiments 2 and 3 in Table 1) similar to those in the parent cell line (3.15 kb), whereas clones containing the antisense vector constructs that underwent crisis had mean TRF lengths of 2.40 and 2.23 kb—that is, they were 27 to 31 percent shorter than those of the vector control clones. These data suggest that telomere repeats were lost in the antisense-containing clones as a result of inhibition of telomerase activity.

To test this directly, we assayed telomerase activity in 14 of the clones in experiment 3. Telomerase activity was generally low but detectable in many of the antisense clones (53), although the presence of shortened telomeres suggests that the amount of activity was not sufficient to maintain telomere length, as the mean TRF fell from 3.22 to 2.23 kb ($P = 0.0004$) in the four clones that went into crisis. In the eight clones that contain the antisense vector (pBBS-hTRb) but did not enter crisis, telomere length was not significantly changed (3.03 versus 3.22; $P = 0.355$), and telomerase activity was similar to that of controls. Taken together, these results support the hypothesis that telomere loss leads to crisis and cell death once telomeres are shortened to a critical length. The induction of cell crisis in HeLa cells expressing antisense hTR demonstrates the potential of telomerase inhibition as a therapeutic approach for treating human cancer.

Fig. 5. Antisense hTR leads to a shorter mean TRF and cell crisis. Three independent experiments were done in which HeTe7 cells (HeLa cells that express large amounts of the tetracycline repressor-VP16 chimeric protein) were transfected by electroporation with either plasmid p10-3-hTR, expressing antisense hTR under control of the tetracycline-VP16-induced cytomegalovirus minimal promoter (54) or pBBS-hTR, expressing antisense hTR under the control of the MPSV promoter (54). [In these experiments, no regulation by tetracycline was observed (57).] As a control, HeTe7 cells were also transfected with the parental vector under the same conditions. All p10-3-hTR and pBBS-hTR clones were shown to express antisense transcripts. (A) TRF analysis is shown for experiment 2. Nuclear DNA from the HeTe7 clones was purified, cut with Hinf I and Rsa I, and run on a 0.5% agarose gel. The DNA was probed in the gel with an [α - 32 P]-labeled (TTAGGG) $_3$ oligonucleotide to label the telomeric TRF. Size markers are shown on the left in kilobases. (B) The mean TRF is shown for each antisense and control clone in experiment 2. The TRF gel was scanned with a Phosphorimager, and the mean TRF quantified as described previously (17). A horizontal line marks the average mean TRF for the antisense and control vector groups.



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34. To enrich for potential telomerase RNAs, we followed two procedures: subtractive hybridization to remove high-abundance common sequences or partial telomerase purification. In the subtraction approach, a PCR-amplifiable cDNA library from telomerase-positive cells was hybridized to an excess of PCR-amplified cDNAs produced from telomerase-negative cells. In this case, total RNA from telomerase-positive 293 cells or telomerase-negative IMR90 cells was size-selected on an agarose gel, made into double-stranded cDNA with reverse transcriptase, ligated to oligonucleotide arms, and amplified by PCR. Control experiments showed that subtraction hybridization of the 293 cell library by the IMR90 cell library depleted the telomerase-positive 293 cell library of most high-abundance common sequences. In the second approach, telomerase from S100 extracts of 293 cells was partially purified with DEAE ion exchange and sephacryl sizing columns, then by sedimentation through a glycerol gradient. A cDNA library was prepared from the total RNA of the peak gradient fractions, ligated to oligonucleotide arms, and amplified by PCR.
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39. DNA was isolated from 293 cells and digested with Bgl II plus Eco RI, Eco RI alone, Eco RI plus Hind III, Hind III alone, Pst I, or Spe I plus Xba I. The restricted DNA was run on an agarose gel, Southern blotted, and probed with an [α -³²P]-labeled TRC3 cDNA fragment of 186 bp. Sizes expected for TRC3 based on the lambda clone (1.0, 4.2, 1.3, >2.4, 0.97, and 1.6) were observed in the genomic Southern blot. This labeled TRC3 probe also specifically hybridized to chromosome 3 in a somatic cell hybrid panel. The chromosome 3 location of the TRC3 was narrowed to the distal end of 3q by chromosome in situ suppression hybridization [I. Parra and B. Windle, *Nature Genet.* **5**, 17 (1993)] with the use of a TRC3-selected genomic lambda clone labeled with biotin-16-deoxyuridine triphosphate (dUTP) as a probe. Chromosome 3 was identified with a chromosome 3-specific centromeric DNA (which was labeled with digoxigenin-11-dUTP) as a probe.
40. RNA was extracted from purified telomerase preparations and was then used in the following experiments. The method of the 5' end mapping was as follows. First-strand cDNA was made with antisense TRC3 primers (R3B: 5'-CAACGAACGGCCAGCAGCTGACATT) in the presence of [α -³²P]dATP. Extension products were resolved by polyacrylamide gel electrophoresis, identified by autoradiography, excised, and extracted. An oligonucleotide (NotA: 5'-pATAGCGGCGGCTTGCCTTCA) was ligated to these first-strand products with T4 RNA ligase, and PCR was then done with a nested primer R3c (5'-GTTTGCTCTAGAATGAACGGTGGGAAG) and an oligonucleotide (NotB: 5'-TGAATTCTTGGCGCCGCTAT) complementary to the NotA oligonucleotide. PCR products were resolved on agarose gels, excised, and sequenced directly with oligonucleotide G1 (5'-AGAAAAACAGCGCGGGGAGCAAGCA) as a primer. The method for the 3' end mapping was as follows. An RT-PCR strategy was first used in which a series of antisense primers complementary to the genomic DNA sequence primed first-strand cDNA synthesis. The primers in this series were spaced at approximately 150-bp intervals starting from the 5' end of the transcript. PCR was then done with the first-strand primer and a primer whose sequence was internal to the known TRC3 transcript (F3b: 5'-TCTAACCTAACTGAGAAGGGCGTAG). Reverse transcriptase-sensitive PCR bands of the predicted size were seen with cDNA made with all primers designed to the interval +96 to +446 but not with any of the primers designed to +554 to +946. TRC3 runs at ~450 bases on Northern blots, which places the putative 3' end of the mature TRC3 transcript at +450, although longer TRC3 transcripts out to +541 have been detected by RT-PCR.
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52. A 200-bp Eco RI DNA fragment containing nucleotides 1 to 185 of TRC3 was inserted into the Eco RI sites of p10-3 and pBBS212 to make the plasmids p10-3-hTR and pBBS-hTR, respectively. Plasmid p10-3-hTR expresses the antisense of hTR under the control of the tetracycline-regulated cytomegalovirus minimal promoter [M. Gossen and H. Buiard, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 5547 (1992)]. Plasmid pBBS-hTR expresses the antisense of hTR under the control of the MPSV promoter (54).
53. Because of the small cell populations, telomerase activity was assayed for the samples in experiment 3 by the PCR method (17), which has limited use as a quantitative assay. HeTe7 cells that expressed the antisense vector and went into crisis had some one-fifth of the telomerase activity found in vector controls. In contrast, those antisense clones that did not go into crisis averaged three-fifths of the telomerase activity found in the control vector clones.
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58. The experiments with p10-3-hTR were done in the absence of tetracycline, because control experiments with luciferase or antisense hTR under the control of the tetracycline-VP16-induced cytomegalovirus minimal promoter showed that the presence or absence of tetracycline had little effect on luciferase or antisense hTR expression in HeTe7 cells. In early experiments with antisense hTR constructs in HeTe7 cells, the cells still underwent crisis at 23 to 26 PDs in the presence of tetracycline.
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